



## **BIOACTIVE COATING FOR MEDICAL DEVICES**

[0001] The present application claims the benefit of U.S. Provisional Patent Application Serial number 60/399,039, filed July 25, 2002, pending.

### **Field of the Invention**

[0002] The present application relates to coatings for medical devices, preferably coatings for medical implants. More particularly, the application relates to bioactive coatings, preferably bioactive coatings comprising keratin having osteoinductive properties.

### **Background of the Invention**

[0003] Many different types of coatings have been developed for purposes of passivating the surface of a medical device. Collagen, hydroxyapatite, and more recently the so-called RGD peptides all have been investigated as coatings in orthopedic implant applications. These materials all have been shown to be at least osteoconductive, but none have been shown to be osteoinductive. Passivating coatings are needed which have osteoinductive properties and which can mask the implant from the human immune system.

### **Brief Summary**

[0004] The present application provides a medical implant comprising a passivating coating comprising keratin. The passivating coating comprising a bonding region and a bioactive region. The said bonding region comprises at least one organosilane compound comprising a silane component bound to a surface of said substrate. The bioactive region comprises an organic component of said organosilane bound to a reactive pendant group on the keratin.

### **Detailed Summary**

[0005] The present application provides substrates comprising a passivating coating comprising keratin. In a preferred embodiment, the passivating coating comprises osteoinductive

properties. In a more preferred embodiment, the passivating coating comprises both osteoinductive properties and osteoconductive properties.

[0006] The substrate preferably resides on a medical device, most preferably a medical implant. The medical device preferably is selected from the group consisting of tissue engineering constructs, orthopedic implants, dental implants, and ventricular assist devices.

[0007] The substrate comprises a biocompatible material. The biocompatible material preferably is selected from the group consisting of silicon, metals, metal alloys, and ceramics. A preferred metal comprises titanium, and may be titanium metal or an alloy thereof. A preferred ceramic comprises hydroxyapatite.

[0008] The passivating coating comprises keratin and, in a preferred embodiment, one or more bioactive factors selected from the group consisting of bone morphogenetic protein (BMP) and transforming growth factor beta (TGF- $\beta$ ). The keratin is derived from a material selected from the group consisting of hair, fur, feathers, horns, hooves, beaks, or feet. The keratin preferably is derived from hair, more preferably from human hair, which may be referred to as "human hair keratin." The keratin also preferably comprises reduced keratin.

[0009] The passivating coating preferably comprises a bonding region and a bioactive region. The bonding region comprises a material adapted to bond with both a surface of the implant and with reactive pendant groups on the keratin. Preferably, the bonding region comprises at least one silane compound, more preferably an organosilane.

[0010] The organosilane preferably comprises a silane component and an organic component. The bonding region comprises the silane component bonded with a surface of the medical device and the bioactive region comprises the keratin bonded to the organic component of the organosilane. Suitable organic components for the organosilane are adapted to react with

reactive pendant groups on the keratin. The organosilanes preferably comprise an organic component comprising a moiety selected from the group consisting of epoxy groups, alkoxy groups, vinyl groups, amine groups, isocyanate groups, and carboxyl groups. Preferred organic components comprise a moiety selected from the group consisting of epoxy groups, alkoxy groups, vinyl groups, and amine groups. More preferred organic components are selected from the group consisting of epoxy groups, acrylate groups, alkoxy groups, vinyl groups, and alkylamine groups. Most preferred organic moieties are selected from the group consisting of vinyl groups and epoxy groups. The organosilane preferably comprises substituents selected from the group consisting of from about 1 to 3 halogens and from about 1 to 3 alkoxy groups. A preferred halogen is chlorine.

**[0011]** Also provided is a method of coating a substrate with a passivating substrate. The method comprises: bonding a coupling material to said substrate, producing a bonding region; and, bonding keratin to said bonding region. The method further preferably comprises oxidizing a surface of said substrate before bonding a coupling material to the substrate.

**[0012]** In a preferred embodiment the substrate is cleaned before bonding the coupling material to the substrate. Most preferably, the cleaning comprises sonication in anhydrous solvent and sonication in water. Suitable anhydrous solvents include, but are not necessarily limited to methanol, ethanol, isopropyl alcohol, dimethylsulfoxide, acetone, or tetrahydrofuran. A preferred anhydrous solvent is dichloromethane. The water preferably is deionized water.

**[0013]** Bonding keratin to said bonding region comprises dissolving keratin in an appropriate solvent and adding an anhydrous solvent to produce a keratin mixture, exposing said bonding region to said keratin mixture; and curing under conditions effective to produce said bioactive region.

[0014] For reduced/reduced keratins, an appropriate solvent is water. For oxidized/reduced keratins, an appropriate solvent comprises an aqueous solution comprising a base. Suitable bases include, but are not necessarily limited to ammonium hydroxide, sodium hydroxide, and/or potassium hydroxide. A preferred base is ammonium hydroxide.

[0015] Suitable anhydrous solvents include, but are not necessarily limited to methanol, ethanol, isopropyl alcohol, dimethylsulfoxide, acetone, or tetrahydrofuran. A preferred anhydrous solvent is dimethylsulfoxide.

[0016] The method further comprises mixing the keratin mixture with a reagent selected from the group consisting of a catalyst and an initiator. When a vinyl-functional silane coupling agent is used, a suitable initiator or catalyst generates free radicals. A preferred free radical initiator is selected from the group consisting of anthraquinone-2-sulfonic acid or anthraquinone-2-sulfonic acid sodium salt monohydrate (Aldrich; Milwaukee, WI).

#### **Brief Description of the Figures**

[0017] Figure 1 is a graph showing the water contact angle of keratin-coated titanium disks, which demonstrates that disks subjected to the process of the present application, silane coating followed by grafting of keratins, had the lowest resulting contact angle.

[0018] Figure 2 is a graph showing the water contact angle of keratin-coated substrates demonstrating that both titanium and glass were effectively coated using the dipping process.

[0019] Figure 3 is a chart comparing XPS spectra between an untreated titanium disk and a) a silane-coated disk (Si2p spectra) and b) a disk treated with silane, catalyst and keratin (N1s spectra).

[0020] Figure 4 is a graph showing Si and N XPS atomic concentrations of keratin-coated titanium disks.

[0021] Figure 5 is the estimated thickness of coatings on titanium disks, where the numbers in parenthesis indicate the thickness of the films on the silane coating.

[0022] Figure 6 is a micrograph showing that 2T3 cells adhere to and differentiate on keratin-coated titanium disks.

### **Detailed Description**

[0023] As a family with more than 100 homologues, the biocompatibility of keratins is predetermined by their structural variation. No other human protein offers this magnitude of structural and compositional diversity, and therefore, tolerance by the immune system.

[0025] The present application selectively extracts keratins from human hair fibers and uses the keratins in a dipping process which produces a molecular coating on the surfaces of medical device materials, such as metals and ceramics. The method uses a coupling agent adapted to bond with both a surface of the implant and with reactive pendant groups on the keratin. Preferably, the bonding region comprises at least one silane compound, more preferably an organosilane. The organosilane preferably comprises a silane component and an organic component, wherein the bonding region comprises the silane component bonded with a surface of the medical device and the organic component bonded with reactive moieties on the keratin. In a preferred embodiment, the silane component of the organosilane is covalently bonded with the surface of the medial device and the organic component of the organosilane is covalently bonded with reactive moieties on the keratin.

[0026] Characterization of these coatings by water contact angle and X-ray photoelectron spectroscopy (XPS) revealed highly altered surface properties. The angstroms-thin keratin coating was robust, and did not interfere with the normal topology of grit blasted titanium disks.

These surface properties were shown to be conducive to the attachment, growth, and differentiation of 2T3 mouse osteoblasts on keratin coated titanium disks in culture.

[0027] The coating method is generally applicable to medical device materials, which typically comprise metal and ceramic surfaces. The coating method has the potential of producing a more biocompatible interface and ultimately, biologically active surfaces that promote host acceptance and/or accelerate tissue on growth.

### **Keratins**

[0028] Human hair is composed of a tough, tubular outer layer made up of flattened cells arranged in a scaly, overlapping profile known as the cuticle. The inner bulk of the hair is known as the cortex and is constructed from elongated cells that are densely packed with fibrous keratins. The fibrous keratins are arranged in bundles, referred to as macrofibrils, that contain microfibrillar coiled-coils of  $\alpha$ -helical keratins. The intermediate filaments (IFs) of this structure are bound together with an to amorphous keratin matrix material. The matrix and IFs vary in function and composition. The matrix is the “glue” that holds the IFs together. This glue is high in sulfur content and is made up of low molecular weight keratins (LMWKs), typically less than 10-15 kDa. The IFs are composed of keratins of relatively low sulfur content but high molecular weight (high molecular weight keratins or HMWKs), generally 50-85 kDa.

[0029] One distinguishing characteristic of keratins is that they are afforded their structural integrity in large part by the presence of disulfide crosslinks which form a three dimensional network of polypeptide chains. This network structure renders keratins insoluble. Keratins can, however, be made soluble by destroying this three-dimensional structure via disulfide bond scission. Disulfide bond scission can be done oxidatively, reductively, or by a combination of the two.

[0030] Disulfide bond scission and selective extraction under controlled conditions results in the isolation of LMWKs from HMWKs. This process serves to remove the cortical proteins without disrupting the cuticle. The inner structure of the hair fiber is removed without substantial damage to the cuticle. The HMWKs from this extraction process give rise to two broad protein bands by gel electrophoresis at molecular weight of 66 kDa and 43 kDa. These keratins are useful as structural biomaterials, including coatings for ceramic and metal surfaces.

[0031] Growth factors, such as bone morphogenetic protein (BMP) and transforming growth factor beta (TGF- $\beta$ ), are present in the hair follicle, are deliverable by the coatings, and provide the coatings with osteoinductive and osteoconductive properties.

[0032] Keratin comprises amino acids, which generally have the formula:

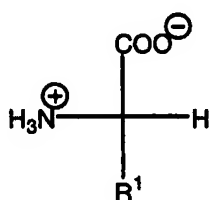
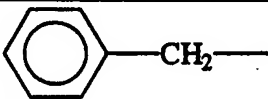
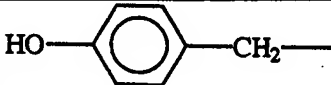


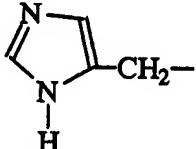
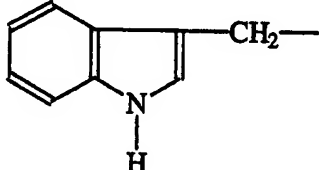
Table 1 summarizes the amino acid residues found in human hair, for example, and shows the “R1” groups associated with each residue.

Table 1. Ranked average amounts of amino acids in human hair

Table 1. Ranked average amounts of amino acids in human hair					
Amino Acid	R <sup>1</sup> Group	Nature	pKa	Isoelectric Point (pH)	Percent Composition in Hair
Cysteine	H—S—CH <sub>2</sub> —	Nonpolar	8.4	5.02	17.3
Glutamic Acid	$\begin{array}{c} \text{O} \\    \\ \text{HO}-\text{C}-\text{CH}_2-\text{CH}_2- \end{array}$	Polar	4.5	3.22	13.9
Arginine	NH	Polar	12.5	11.15	9.85

	$\begin{array}{c} \text{NH}_2-\text{C}-\text{N}-(\text{CH}_2)_3- \\    \\ \text{H} \end{array}$				
Serine	$\text{HO}-\text{CH}_2-$	Polar	None	5.68	9
Threonine	$\begin{array}{c} \text{OH} \\   \\ \text{CH}_3-\text{CH}- \end{array}$	Polar	None	5.64	7.75
Leucine	$\begin{array}{c} \text{CH} \\ \diagdown \\ \text{CH}-\text{CH}_2- \\ \diagup \\ \text{CH}_3 \end{array}$	Hydro-phobic	None	5.98	7.35
Proline	$\begin{array}{c} \text{CH}_2 \\ \diagup \quad \diagdown \\ \text{CH}_2 \quad \text{CH}_2 \end{array}$	Hydro-phobic	None	6.3	6.95
Aspartic Acid	$\begin{array}{c} \text{O} \\    \\ \text{HO}-\text{C}-\text{CH}_2- \end{array}$	Polar	4.5	2.77	5.8
Valine	$\begin{array}{c} \text{CH}_3 \\ \diagdown \\ \text{CH} \\ \diagup \\ \text{CH}_3 \end{array}$	Hydro-Phobic	None	5.96	5.7
Isoleucine	$\begin{array}{c} \text{CH}_3 \\   \\ \text{CH}_3-\text{CH}_2-\text{CH}- \end{array}$	Hydro-phobic	None	5.94	4.75
Glycine	$\text{H}-$	Nonpolar	None	5.65	4.15
Phenylalanine		Hydro-phobic	None	5.48	3
Alanine	$\text{CH}_3-$	Hydro-phobic	None	6	2.8
Tyrosine		Hydro-phobic	None	5.66	2.6
Lysine	$\text{NH}_2-(\text{CH}_2)_4-$	Polar	10.4	9.59	2.5



Histidine		Aromatic	6.2	7.47	0.9
Methionine	$\text{CH}_3\text{—S—CH}_2\text{—CH}_2\text{—}$	Hydrophobic	None	5.74	0.85
Tryptophan		Hydrophobic	None	5.89	0.85

The most abundant amino acid in human hair is cysteine, which is found in the form of disulfide-bridged cystine groups. As discussed above, this group can be converted to other sulfur containing moieties, most notably thiol. Thiols theoretically can be reacted with reactive ends of the coupling material using a number of chemical techniques, such as those described in S. Patai (Ed.), *the Chemistry of the Thiol Group*, Parts 1 and 2, John Wiley & Sons, New York, NY (1974), incorporated herein by reference. Other reaction scenarios, such as those directed toward polymer synthesis, also are useful to utilize thiols to form an assortment of desirable crosslinks, including those described in Rempp, P. and Merrill, E. W., *Polymer Synthesis*, Huethig & Wepf Verlag Basel, Heidelberg, Germany (1986); Young, R. J. and Lovell, P. A., *Introduction to Polymers*, Chapman & Hall, London (1991); Odian, G., *Principles of Polymerization*, John Wiley & Sons, New York, NY (1991), incorporated herein by reference.

[0033] In addition to cysteine, the following amino acids have pendant groups comprising nitrogen or oxygen which may be useful as reactive pendant groups; arginine, serine, glutamic acid, threonine, aspartic acid, lysine, asparagine, glutamine, tyrosine, tryptophan, and histidine. Where the protein is  $\alpha$ -keratin, preferred amino acid residues comprising reactive pendant groups

for crosslinking are cysteine, arginine, serine, and glutamic acid, most preferably cysteine and arginine. Disulfide bond scission and keratin extraction

[0034] The keratins may be processed and/or isolated in a number of ways. Preferably, the processing is sufficient to render the resulting proteins water soluble. Suitable processing techniques include known oxidation techniques, reductive techniques, and/or combinations thereof, as long as the processing renders the proteins water soluble without significant hydrolysis of peptide bonds.

[0035] A number of reductive chemistries are known for disulfide bond scission in keratins: See Wardell, J. L., "Preparation of Thiols" in *The Chemistry of the Thiol Group*, Patai, S. (Editor), pp. 163-353, John Wiley & Sons, New York, NY (1974), incorporated herein by reference. HMWK's may be extracted from hair using at least two reductive extractions, as described in Crewther, W. G., Fraser, R. D. B., Lennox, F. G., and Lindley, H., "The Chemistry of Keratins" in *Advances in Protein Chemistry*, Anfinsen, C. B., Jr., Anson, M. L., Edsall, J. T., and Richards, F. M. (Editors), Academic Press, New York, pp. 191-346 (1965), incorporated herein by reference.

[0036] The following methods are suitable for processing keratins for use in producing the coatings:

#### **-Oxidation/Reduction**

[0037] In a preferred embodiment, which uses keratins as a source material (e.g. human hair), the hair is oxidized by a suitable oxidizing agent. Suitable oxidizing agents include, but are not necessarily limited to hydrogen peroxide, peracetic acid, percarbonates, persulfates, chlorine dioxide, sodium and calcium peroxides, perborates, and hypochlorite. A most preferred oxidizing agent is hydrogen peroxide. The oxidants are used at a concentration of up to about 35%,

preferably at from about 0.1% to about 10%. The oxidation preferably occurs at reflux temperatures.

[0038] In a preferred embodiment, the hair is treated with hydrogen peroxide ( $H_2O_2$ ), at from about 0.1% to about 10%, most preferably 1%, in order to disrupt the cuticle and swell the keratin source material. This process also converts some fraction of the cystine residues into sulfonic acid groups. The amount of oxidation may be controlled by varying the time of oxidation, preferably from about 0 hours to about 4 hours, while retaining the other conditions of the oxidation reaction constant. These conditions include concentration and type of oxidant, temperature, and ratio of extracting media to keratin source material. After the reaction is complete, the oxidized hair is filtered and rinsed, preferably with deionized water. The filtrate is discarded and the hair allowed to dry.

[0039] Where other conditions of oxidation are maintained constant, the conversion rate of cystine to sulfonic acid residues is roughly proportional to the amount of time used for the oxidation. Residual cystines in the resulting oxidized keratin solids are converted to other sulfur-containing moieties using reductive techniques. Preferably, the disulfide-bridged cystine group is converted to a thiol group, which has utility of its own, or can be modified using a variety of chemical techniques.

[0040] The oxidized hair preferably is treated with a reducing agent. Suitable reducing agents include, but are not necessarily limited to thioglycolic acid and salts thereof, mercaptoethanol, dithiothreitol, thioglycerol, thiolactic acid, glutathione, cysteine, sodium sulfide, and sodium hydrosulfide. Preferred reducing agents are thioglycolic acid and mercaptoethanol, most preferably thioglycolic acid.

[0041] In order to treat the oxidized hair with the reducing agent, the previously oxidized hair is suspended in the reducing agent typically at a concentration of up to about 10N, preferably from about 0.1N and 1N; at a pH greater than about 7, preferably equal to or greater than 9, most preferably 9; a temperature of from about 25 to about 80 °C, preferably about 60 °C, preferably for a time period of from about 1 to about 72, most preferably about 24 hours. The reaction occurs under an inert atmosphere, preferably nitrogen. The liquid fraction is separated from any remaining solids using known means, including but not necessarily limited to filtration, or cannulation and/or centrifugation, preferably under inert atmosphere. A preferred method of separation is filtration. Once the solids are removed, the soluble keratin proteins are isolated from the solution by addition of a water-miscible non-solvent, or by spray drying. Water-miscible non-solvents include, but are not necessarily limited to ethanol, methanol, isopropyl alcohol, tetrahydrofuran, acetone, dioxane, and the like, again under inert atmosphere. A preferred non-solvent is ethanol. The precipitate is separated from the non-solvent using known means, preferably by filtration and rinsing using additional aliquots of the non-solvent. The resulting keratin proteins are dried using known techniques, preferably overnight under vacuum at room temperature. This process results in the keratins having both sulfonic acid groups and thiol groups.

[0042] In a most preferred reaction, clean, dry human hair is treated with hydrogen peroxide with heating, preferably at reflux, for a period of time effective to achieve scission of disulfide bonds, typically about 180 minutes. The hair is separated from the liquid, preferably by filtration, and the liquid is discarded. The hair was rinsed with water, preferably in copious amounts, and allowed to air dry. The dried, oxidized hair is then treated with a reducing agent, preferably 1M thioglycolic acid at pH 9 (adjusted with ammonium hydroxide), and the mixture is heated,

preferably to about 60 °C, under inert gas, preferably under a nitrogen atmosphere, for a period of time, preferably 24 hours. After reductive extraction, the solids are separated from the liquid, preferably by centrifugation. The filtered liquid is added, preferably dropwise, to an excess of ethanol, preferably about an 8-fold volume excess of ethanol, thereby forming a keratin precipitate. The precipitated keratins are isolated by filtration and dried under vacuum. The keratin is ground into a fine powder using a mortar and pestle.

#### **Reductive/reductive extraction**

[0043] In another embodiment, a first reductive extraction is performed by treating the hair with a first reducing agent under first conditions effective to selectively extract matrix keratins, producing a first solution comprising soluble reduced matrix keratins (LMWK's) and remaining hair solids (HMWK's). Although it may be possible to subject the LMWK's to the techniques described herein to produce coatings, preferred proteins for use in the techniques herein are HMWK's, which preferably are isolated during a second extraction. The remaining hair solids and the first solution are separated, and the remaining hair solids are exposed to a second extraction solution under second conditions effective to solubilize a-keratins, producing a second solution comprising soluble reduced a-keratins (HMWK's) and solid cuticle.

[0044] Suitable reducing agents again include, but are not necessarily limited to thioglycolic acid and salts thereof, mercaptoethanol, dithiothreitol, thioglycerol, thiolactic acid, glutathione, cysteine, sodium sulfide, and sodium hydrosulfide. Preferred reducing agents are thioglycolic acid and mercaptoethanol, most preferably thioglycolic acid.

[0045] In order to selectively reduce and extract the desired proteins, the hair (or other protein source) is suspended in a reducing agent at a concentration of from about 0.1M to about 10M, preferably about 1.0M. Gentle swelling of hair fibers is achieved at a pH of about 9 or

more, preferably at a pH of from about 9 to about 10.5. Hence, the initial reduction takes place at a temperature of from about 20 to about 100 °C, preferably at about 25 °C. The time period required to accomplish the first reduction is from about 8 to about 36 hours, most preferably about 24 hours. The reaction occurs under an inert atmosphere, preferably nitrogen. The liquid fraction is separated from remaining solids using known means, including but not necessarily limited to filtration, cannulation, and/or centrifugation, preferably under inert atmosphere. A preferred method of separation is filtration.

[0046] A second extraction is performed using a suitable swelling agent, preferably urea, and/or a base such as ammonium hydroxide, sodium hydroxide, or potassium hydroxide. A most preferred swelling agent for this second extraction is concentrated urea. The second extraction effectively removes the fibrous  $\alpha$ -keratins from inside the cuticle. The second extraction occurs at from about 1M to about 10M urea, preferably about 7M urea, for a period of at least about 1 hour, preferably from about 1 to about 72 hours, more preferably 20 hours or more, most preferably about 24 hours. The second extraction occurs at room temperature, but may take place at temperatures of from about 20 °C to about 100 °C, preferably about 25 °C. The liquid fraction is separated from the empty, intact cuticle, using known means. Suitable means include but are not necessarily limited to filtration, cannulation and/or centrifugation, preferably under inert atmosphere. A preferred method of separation is filtration.

[0047] Once the cuticle is removed, the water soluble keratin proteins may be retained in solution for further use, or they may be isolated from the solution by addition to a water-miscible non-solvent, or by spray drying. Water-miscible non-solvents include, but are not necessarily limited to ethanol, methanol, isopropyl alcohol, tetrahydrofuran, acetone, dioxane, and the like, again under inert atmosphere. A preferred non-solvent is ethanol. The precipitate is separated

from the non-solvent using known means, preferably by filtration and rinsing using additional aliquots of the non-solvent. The precipitated proteins are dried using known techniques, preferably overnight under vacuum at room temperature. The extracted water soluble keratin proteins (herein sometimes collectively referred to as “water soluble proteins”) comprise thiols or thiol groups.

### **Coating Process**

#### **-Cleaning the surface**

[0048] Before coating the substrate, the surface is cleaned using any suitable procedure. An example of a suitable procedure is sequential sonication in an anhydrous solvent and water. Suitable anhydrous solvents include, but are not necessarily limited to methanol, ethanol, isopropyl alcohol, dimethylsulfoxide, acetone, or tetrahydrofuran. A preferred anhydrous solvent is dichloromethane. The water preferably is deionized water, followed by drying, preferably in air.

#### **Forming the Bonding Region**

[0049] After cleaning, the implant is immersed in a solution of the coupling material, preferably an organosilane. The use of silane compounds in surface coatings is a well-known industrial process. Arkels B. “Tailoring surfaces with silanes.” *Chemtech* 1977;7:766-778, incorporated herein by reference. Not only can organosilanes passivate and change the chemistry of a surface, they can be used as coupling agents to facilitate the bonding of yet another compound to the outside surface of a substrate.

[0050] There are many different types of commercially available organosilanes with a variety of chemical functionalities. The organosilanes preferably comprise an organic component comprising a moiety selected from the group consisting of epoxy groups, alkoxy groups, vinyl

groups, amine groups, isocyanate groups, and carboxyl groups. Preferred organic components comprise a moiety selected from the group consisting of epoxy groups, alkoxy groups, vinyl groups, and amine groups. More preferred organic components are selected from the group consisting of epoxy groups, methacrylate groups, alkoxy groups, vinyl groups, and alkylamine groups. Most preferred organic moieties are selected from the group consisting of vinyl groups and epoxy groups.

**[0051]** The organosilane preferably comprises substituents selected from the group consisting of from about 1 to 3 halogens and from about 1 to 3 alkoxy groups. A preferred halogen is chlorine.

**[0052]** A preferred organosilane, particularly where the surface is titanium, is vinylmethyldichlorosilane (Gelest Inc., Tullytown, PA). The organosilane is in an organic solvent. The organic solvent may be substantially any organic solvent that is effective to maintain the organosilane in solution without negatively impacting the bonding process. The organic solvent preferably comprises elements other than oxygen and/or nitrogen. Examples of suitable organic solvents include, but are not necessarily limited to alkanes, alkylene chlorides, chloroform, xylenes, and combinations thereof. A preferred alkane is hexane and a preferred alkylene chloride is methylene chloride. A most preferred organic solvent is hexane. The percentage of the organosilane in the organic solvent may vary from about 1 to about 10 weight percent, preferably about 1 weight percent.

**[0053]** The substrate is maintained in the solution for a time sufficient to form the coating, suitably from about 1 to about 30 minutes, typically about 10 minutes. The substrate then is removed from solution, rinsed with copious amounts of fresh organic solvent, and allowed to air dry.



### **-Forming the bioactive region**

[0054] To form the bioactive region, keratin powder obtained as described above is dissolved in an appropriate solvent. For reduced/reduced keratins, an appropriate solvent is water. For oxidized/reduced keratins, an appropriate solvent comprises an aqueous solution comprising a base. Suitable bases include, but are not necessarily limited to ammonium hydroxide, sodium hydroxide, and/or potassium hydroxide. A preferred base is ammonium hydroxide. In a preferred embodiment, about 15 grams of the keratin powder is dissolved in about 30 mL of 3N ammonium hydroxide.

[0055] An anhydrous solvent is added. Suitable anhydrous solvents include, but are not necessarily limited to methanol, ethanol, isopropyl alcohol, dimethylsulfoxide, acetone, or tetrahydrofuran. A preferred anhydrous solvent is dimethylsulfoxide. At the foregoing weight and volume of keratin and base, about 270 mL of dimethylsulfoxide is added. The mixture is stirred with mild heating until the keratin is dissolved.

[0056] A suitable catalyst or initiator preferably is used. For example, wherein a vinyl-functional silane coupling agent is used, a suitable initiator or catalyst is used to generate free radicals. An example of such an initiator is a UV activated photoinitiator, such as anthraquinone-2-sulfonic acid or anthraquinone-2-sulfonic acid sodium salt monohydrate (Aldrich; Milwaukee, WI). At the foregoing weights and volumes, about 1.5 grams of anthraquinone-2-sulfonic acid sodium salt monohydrate is added. The resulting solution is poured over the silane-coated implant such that it is completely submerged. The coating on the submerged substrate then is cured using an effective energy source, such as a heat lamp, an autoclave, a microwave, or a UV lamp. A preferred energy source is a UV lamp ( $\lambda=365$  nm, 1.05 amps). Curing is continued for a sufficient period of time to cure the coating, typically from about 1 to about 24 hours, preferably

for about 24 hours. After curing, the substrate is removed from the solution, rinsed, preferably with copious amounts of deionized water, and allowed to dry, preferably by air.

[0057] Persons of ordinary skill in the art will recognize that the foregoing parameters, such as weights, volumes, and times, may be varied depending upon the surface area of the implant, the particular bonding material used, and other factors.

[0058] The application will be better understood with reference to the following examples, which are illustrative only, and should not be construed as limiting the claims to a particular embodiment. The following materials and methods were used in the examples described below:

### **Materials and Methods**

#### *Preparation of Keratins*

[0059] In a typical reaction, 500 g of clean, dry human hair was placed in a 12 L round bottom flask. 8,350 mL of 1 weight/volume percent of hydrogen peroxide was added and the reaction heated to reflux for 180 minutes. The hair was separated from the liquid by filtration and the liquid discarded. The hair was rinsed with copious amounts of water and allowed to air dry. 100 g of the dried, oxidized hair was placed in a 2000 mL round bottom flask. 1000 mL of 1M thioglycolic acid at pH 9 (adjusted with ammonium hydroxide) was added and the mixture was heated to 60°C under a nitrogen atmosphere for 24 hours. After reductive extraction, the solids were separated from the liquid by centrifugation. The filtered liquid was added dropwise to an 8-fold volume excess of ethanol, thereby forming a keratin precipitate. The precipitated keratins were isolated by filtration and dried under vacuum. The keratin was ground into a fine powder using a mortar and pestle.

#### *Titanium disks*

[0060] Grit blasted titanium test disks with highly controlled and thoroughly characterized surfaces to be used for cellular response were prepared. The material chosen was a medical grade, commercially pure titanium (C.P. Ti). The materials were purchased as large sheets (approximately 5 ft. x 4 ft. x 0.050 in., to ensure metallurgical homogeneity for all sample sets) from Timet (O'Fallon, MO, USA).

[0061] 1 ft. x 1 ft. sheets were cut from the original large single sheet. In order to ensure a consistent surface finish for each disk, surface preparation was performed on these sheets. Each 1 ft. x 1 ft. sheet was subjected to the aluminum oxide grit blasting process with compressed air using a #20 grit aluminum oxide (Al<sub>2</sub>O<sub>3</sub>) powder for the rough surface finish; grit blasting was performed by Southwest Research Institute (San Antonio, TX, USA).

[0062] Quality control of the surface finishing operations was performed and gauged by the consistency of surface roughness measurements. The surface roughness was measured using a diamond stylus contact profilometer at 10 different locations on each 1 ft. x 1 ft. plate. The roughness measurements were taken in all directions. The average Ra was 9.56  $\mu\text{m}$  with a standard deviation of 1.63  $\mu\text{m}$ .

[0063] Disks with 14.75 mm diameter and 0.80mm thickness were prepared for the cell assays. The disks were washed 3 times in a detergent solution and rinsed 2 times with DI water in between each wash. After the final rinse, ethanol was poured over the disks to remove excess water. The disks were air-dried.

#### *Coating of Titanium Disks*

[0064] In a typical procedure, titanium disks were cleaned by sequential sonication in dichloromethane and deionized water followed by drying in air. Disks were immersed in a 1 weight percent solution of vinylmethyldichlorosilane (Gelest Inc., Tullytown, PA) in hexanes for

10 minutes. Each disk was individually removed from solution, rinsed with copious amounts of fresh hexanes, and allowed to air dry.

[0065] In a typical keratin coating step, 15 grams of the keratin powder was dissolved in 30 mL of 3N ammonium hydroxide. 270 mL of dimethylsulfoxide was added and the mixture stirred with mild heating until the keratin dissolved. 1.5 grams of anthraquinone-2-sulfonic acid sodium salt monohydrate (Aldrich; Milwaukee, WI) was added and the solution poured over the silane-coated titanium disk samples such that they were completely submerged in a shallow glass dish. The glass dish was placed under a UV lamp ( $\lambda=365$  nm, 1.05 amps) for 24 hours. After UV exposure, each disk was removed from solution, rinsed with copious amounts of deionized water, and allowed to air dry.

#### *Water Contact Angle*

[0066] Contact angles were measured on an optical comparator by placing a 100- $\mu$ L drop of ultrapure water on the horizontal substrate. Interfacial energies were allowed to equilibrate for 30 seconds prior to obtaining a measurement.

#### *X-ray Photoelectron Spectroscopy (XPS)*

[0067] XPS data was obtained using a Physical Electronics PHI5700 ESCA system with an Al monochromatic source (Al K $\alpha$  radiation at 1486.6 eV). The base pressure in the XPS ultrahigh vacuum chamber was  $2 \times 10^{-10}$  torr during the analysis. High resolution scans were obtained with a step size of 0.1 eV and pass energy of 11.75 eV. The presence of the silane and keratin coatings was monitored by tracking the atomic concentration of elements that are only present in each of these coatings. Silicon was used for the silane coating and nitrogen for the keratin. Although sulfur is also present in keratin and detected by XPS, it was not used in this

work as the signal-to-noise ratio for sulfur was close to 1.5; therefore, the atomic concentrations calculated for the sulfur signal were in the same order as the error bars.

[0068] XPS was also used to estimate the film thickness. If the signal of a clean substrate is known, then the XPS signal from the substrate with a film on it can be calculated using the following standard attenuation equation

$$N = N_o \cdot \exp[-t / (\lambda \cdot \sin\theta)]$$

where  $N$  is the XPS signal (area under the peak) of the substrate with a film on it,  $N_o$  is the XPS signal of the clean substrate,  $t$  is the thickness of the film,  $\lambda$  is the mean-free path of photoelectrons in the film (assumed to be 20 Å in this work), and  $\theta$  is the angle between the XPS detector and the surface, equal to 45 degrees in this case. C. S. Fadley, *Progress in Surface Science* 1984;16: 275-388, incorporated herein by reference.

#### *Osteoblast Cell Culture Assay*

[0069] Alpha-minimal essential medium ( $\alpha$ -MEM) was purchased from Gibco BRL (Grand Island, NY, USA) and fetal bovine serum (FBS) was from Summit Biotechnology (Fort Collins, CO, USA). Formalin was obtained from Electron Microscopy Sciences (Fort Washington, PA, USA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

[0070] Driven by the bone morphogenetic protein 2 (BMP-2) promoter, the 2T3 cell line has been isolated and cloned from a transgenic mouse. Ghosh-Choudhury N, Windle JJ, Koop BA, Harris MA, Guerrero, DL, Wozney JM, Mundy GR, and Harris SE. "Immortalized murine osteoblasts derived from BMP2-T-antigen expressing transgenic mice." *Endocrinology* 1996;137:331-339, incorporated herein by reference.

[0071] These cells undergo bone matrix formation *in vitro*. 2T3 cells were plated onto each titanium disk in a 12-well plate, one plate per group. The cells were cultured with  $\alpha$ -MEM supplemented with 10% FBS until they reached 90% confluence, approximately day 5. The

medium was changed every other day. Once the cells reached confluence, the medium was changed to  $\alpha$ -MEM containing 5% FBS, 100  $\mu$ g/ml ascorbic acid (AsA) and 5 mM ( $\beta$ -glycerophosphate ( $\beta$ -GP) for the treatment group or to 5% FBS  $\alpha$ -MEM without AsA and  $\beta$ GP for the control group. The medium was changed every three days and fresh reagents were added up to day 14, which was the end of the treatment period. Cells were cultured at 37°C, 5% CO<sub>2</sub>.

#### *Von Kossa Staining*

[0072] The von Kossa cell staining was modified from a previously published procedure. Beresford JN, Graves SE, and Smoothy CA. "Formation of mineralized nodules by bone derived cells in vitro: a model of bone formation?." *Am. J. Med. Genet.* 1993;45:163-78.

[0073] To fix the 2T3 cells onto the titanium disks, the cells were first washed with PBS and fixed in 10% phosphate buffered formalin for 10 min. The formalin solution was aspirated and the cells were rinsed with deionized water (dH<sub>2</sub>O). Fresh 2% silver nitrate (AgNO<sub>3</sub>) was added to the cells and the titanium disks with cells were then put under sunlight for 10 minutes. After 10 minutes, the AgNO<sub>3</sub> was removed and the 2T3 cells were rinsed under a steady stream of dH<sub>2</sub>O. A 5% sodium thiosulfate (Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) solution was added to the cells for 3 minutes. The Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> was removed and the cells were rinsed again with dH<sub>2</sub>O. The following ethanol (EtOH) series was applied to the cells: 95% EtOH for 30 sec., repeat once more followed by two 100% EtOH applications for 30 seconds each. The cells were then allowed to air dry.

#### *Scanning electron microscopy*

[0074] To obtain images of the surface morphology, the titanium disks with cells were gold/palladium (Au/Pd) coated for 10 seconds in a sputter coater. Images of the titanium surface were obtained on an Amray scanning electron microscope (SEM) (Amray, Bedford, MA, USA) at 15 kV. Images were acquired at three magnifications: 100X, 500X, and 1,000X.

### *Statistical Methods*

[0075] Where indicated, differences between sample groups were determined using a two-tailed Student's t-Test with unequal variance. Statistical significance was established at  $p$  values of less than 0.05.

### **Example 1**

[0076] In the present study, a vinyl-functional silane coupling agent was used to graft keratins onto coated glass and titanium substrates using free radical addition chemistry. A UV activated photoinitiator, anthraquinone-2-sulfonic acid, was used to generate free radicals, although thermal initiation with another catalyst could also be used. In the first step of this process, excellent coating of the substrates was achieved by dipping into a hexane solution of the silane. As shown in Figure 1, the resulting increase in water contact angle for titanium disks averaged 38 degrees. The treatment groups included various combinations of keratin coatings with and without the silane coupling, both in the presence and absence of the photoinitiator (designated as "cat." in Figure 1). This was done to ascertain the effectiveness of the two step coating approach. Although all of the sample groups resulted in a contact angle different than that of the control titanium group (statistical significance of  $p \leq 0.05$ ), the full treatment of silane coating followed by covalent grafting of keratin resulted in the most impressive reduction in contact angle compared to the untreated control. The fully treated titanium disk samples had an average contact angle of 7.6 degrees. The results for glass substrates were similar with a reduction in contact angle between the untreated control and the fully treated sample of 16.5 degrees. The keratin treated glass had an average contact angle of 9.0 degrees. These data are shown in Figure 2. Once it was determined that both titanium disks and glass microscope slides could be coated, only the titanium disks were used in further evaluations.

[0077] As mentioned previously, the successful coatings were achieved by dipping the titanium disks into a hexanes solution of the silane. The XPS Si2p signals from the untreated titanium disk and a silane-coated titanium disk are shown in Figure 3a. The Si signal from the coated disk corresponded to an atomic concentration of ~20%, which compared to <3% for the untreated disk clearly indicates the presence of the silane coating. The small amount of Si on the untreated disks was likely due to surface contamination. The addition of keratin resulted in an increase of nitrogen atomic concentration from ~3 % (background level on an untreated disk) to 8.5%. Figure 3b shows the typical N1s XPS signal from an untreated titanium disk and that of the sample coated with silane, the catalyst and keratin. The atomic concentrations of Si and N measured on the different treatment groups are shown in Figure 4. The concentration of these two elements correlated very nicely with the presence of the organosilane and the keratin.

[0078] The addition of the catalyst and keratin on the silane coating resulted in a decrease in the atomic concentration of Si, which results from the attenuation of the silicon signal by the different additional coatings. The titanium XPS signal was used in the attenuation equation described above to calculate the overall film thickness. The results are shown in Figure 5. The variations in the thickness of the films with silanes + “cat” or keratin are probably due to variations in the thickness of the silane film itself. Nevertheless, it is clear from the cat + keratin film and the keratin-only film that the keratin film is more robust in the presence of the silane coating.

[0079] In order to exclude variations in the silane film and to more accurately determine the thickness of the keratin coating, the attenuation equation was used, treating the silane as the substrate and using the silicon signal from silane-coated disks to estimate the thickness of the keratin coatings on top of the silane film. The Si2p XPS signal from the disk coated only with



silane was used as the clean substrate signal in this case. The results for several different samples are indicated in parentheses in Figure 5. The data indicates that the average thickness of the keratin coatings is  $\sim 8.5 \text{ \AA}$ .

[0080] Differentiation and mineralization of osteoblasts on keratin coated titanium disks were demonstrated by von Kossa analysis of the disk surfaces. Nodule formation is considered to be an important step toward mineralization *in vitro*.

[0081] Cells were cultured in a-MEM containing 5% PBS in the presence (+) or absence (-) of 100  $\mu\text{g/ml}$  ascorbic acid and 5 mM  $\beta$ -glycerophosphate. Differentiation and mineralization of osteoblasts as demonstrated by von Kossa analysis (panels A and C) without (-) AsA and  $\beta$ -GP showed smaller and fewer nodules. After 2 weeks, 2T3 cells were observed to mineralize larger nodules on the implant surface in the presence (+) of 100  $\mu\text{g/ml}$  AsA and 5mM  $\beta$ -GP (panels B and D). Images A and B were acquired at 100X magnification (scale bar = 100 $\mu\text{m}$ ) and C and D at 1,000X magnification (scale bar = 10 $\mu\text{m}$ ).

[0082] The mineralization of nodules *in vitro* appears to resemble the *in vivo* produced woven bone. After 2 weeks, 2T3 cells were observed to mineralize in culture on the implant surface with or without AsA and 13-GP. Without AsA and  $\beta$ -GP, von Kossa staining of the cells at 2 weeks showed smaller and fewer nodules (Figure 6A and C). In contrast, there were more and larger nodules on AsA and  $\beta$ -GP treated disks (Figure 6B and D). The ability of AsA and  $\beta$ -GP to induce nodule formation in 2T3 osteoblasts on collagen coated substrates has been previously reported. In this study, we find similar results for keratin coated substrates, thus indicating biocompatibility.

[0083] The two step dipping process for applying a keratin coating onto titanium substrates resulted in films that were hydrophilic and robust as evidenced by the water contact angle and

XPS data, respectively. This process should result in a more biostable coating as the keratin is covalently bonded to the surface. The coating process resulted in very thin layers of both silane and keratin and would not be expected to interfere with the surface topology often employed by implant manufacturers to promote cell attachment to roughened titanium implant surfaces. The 2T3 osteoblast assay showed that the coating promoted attachment, growth, and differentiation. The ability of these cells to produce bony matrix is demonstrative of the keratin coatings osteoconductive properties. In future studies we intend to demonstrate that these keratin coatings are capable of accelerating the rate of bone on growth.

**[0084]** Persons of ordinary skill in the art will recognize that many modifications may be made to the foregoing without departing from the spirit and scope thereof. The embodiment described herein is meant to be illustrative only and should not be taken as limiting the invention, which is defined in the following claims.